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# ISOLATION AND SCREENING OF PRODIGIOSIN PRODUCING BACTERIA AND CHARACTERIZATION OF PRODUCED PIGMENT

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**ABSTRACT**
Biopigment are natural compounds (secondary metabolite) produced by many organisms represent one of the important sources of potential lead compounds. Prodigiosin (5[(3-methoxy-5-pyrrol-2-ylidene-pyrrol-2-ylidene) is a red, tripyrrole, water insoluble, bioactive pigment produced by number of different bacteria, actinomycetes and some fungi. Kraft in 1902 named red colour compound which he extracted from *Bacillus prodigiosus* as Prodigiosin. Wrede and Hettche (1929) obtained the Prodigiosin from bacterium *S. marcescens* using sodium hydroxide treatment process. In the present study five different samples (starchy food, clinical, milk, water and Soil) were used for isolation of pigment producing bacteria. Four out of sixteen characteristic red colour bacterial isolate obtained shows notable yield of Prodigiosin in peptone broth was selected for further study. The selected strains were identified by using morphological and biochemical analysis. The produced pigment was further characterized by using spectroscopic analysis (by comparing its lambda max with the standard.) and by using chromatographic studies (by comparing its Rf value with the standard.).

**KEY WORDS:** Prodigiosin, Red pigment, *Serratia Spp.*, Biochemical Analysis.

## INTRODUCTION

The plant and microorganism are the two potent source of Biopigment (Papa Georgiou *et al*., 1979, Cho *et al*., 2002). Although pigments from the micro-organisms have been preferred over those from plants because of their stability (Raisainen *et al*., 2002) and the availability of their cultivation technology (Kim *et al*., 1999; Parekh *et al*., 2000) throughout the year. From *Bacillus prodigiosus* red pigment was isolated and named "Prodigiosin" by Kraft in 1902. Raunditz (1933), Wrede and Rothhaas (1933-1934), Wrede and Hettche (1929), Wrede (1930 and 1932), uncover the chemistry of the molecule and defined the structure as a tripyrrole methane. Bacteria are present everywhere in nature, they made a large domain of unicellular, prokaryotic microorganisms which can be identified and classified by using their biochemical, morphological and molecular characteristics. *Serratia marcescens* is a facultative anaerobic, motile, nonspore forming, rod shaped, Gram-negative bacteria of family Enterobacteriaceae. This bacterium produces characteristic red or orange pigment which now a day's used in number of industrial, environmental and pharmaceutical processes (Patton *et al*., 2000). Spectrum of application of Prodigiosin has widened in many sectors such as it is of potential clinical interest because it is reported to have distinct biological activities like antibacterial, antifungal, antiprotozoal, (Croft, *et al*., 2002). Cytotoxic, algicidal (Nakashima *et al.*, 2005) antitumor (Castro, 1967), (Perez-Tomas *et al*., 2003), antimalarial, antidiabetes, antioxidants, immunosuppressive, non-steroidal anti-inflammatory drugs with immune modulatory activities (Alonzo V. *et al*., 1997). Ultraviolet–visible spectroscopy is the absorption spectroscopy or reflectance spectroscopy, which use UV-visible light. The absorption or reflectance in the visible range directly affects by the perceived color of the chemicals involved. This technique is complementary to fluorescence spectroscopy, in that fluorescence deals with transitions from the excited state to the ground state, while absorption measures transitions from the ground state to the excited state (Skoog, *et al.,* 2007). Spectrophotometry is generally performed by using monochromatic light. Because the extent to which a sample absorbs light depends strongly upon the wavelength of light. The spectrum of absorbance shows how the absorbance of light depends upon the wavelength of the light. The spectrum itself is a plot of absorbance vs wavelength and is characterized by the wavelength ($\lambda_{max}$) at which the absorbance is the greatest.

The value of $\lambda_{max}$ is important for several reasons. This wavelength is characteristic of each compound and provides information on the electronic structure of the analyte. In order to obtain the highest sensitivity and to minimize deviations from Beer's Law analytical measurements are made using light with a wavelength of $\lambda_{max}$. The discovery of paper chromatography in 1943 by Martin and Synge provided, for the first time, the means of surveying constituents of plants and for their separation and identification (Martin, A.J.P. *et al.,* 1943). Paper chromatography is an analytical method that is used to

separate colored chemicals or substances, especially pigments. This can also be used in secondary or primary colors in ink experiments. This method has been largely replaced by thin layer chromatography, but is still a powerful teaching tool (Martin A.J.P.*et al.,* 1944). The retention factor ($R_f$) may be defined as the ratio of the distance traveled by the substance to the distance traveled by the solvent. $R_f$ values are usually expressed as a fraction of two decimal places. If $R_f$ value of a solution is zero, the solute remains in the stationary phase and thus it is immobile. If $R_f$ value is 1 then the solute has no affinity for the stationary phase and travels with the solvent front. To calculate the $R_f$ value, take the distance traveled by the substance divided by the distance traveled by the solvent (as mentioned earlier in terms of ratios). $R_f$ value depends on temperature and the solvent used in experiment, so several solvents offer several $R_f$ values for the same mixture of compound. So in the present study produced pigment from the selected bacterial isolate was characterized by Spectrophotometric and chromatographic studies.

## MATERIALS & METHODS

### Collection of different samples and their microbiological analysis

#### Starchy food sample

Food samples having high percentage of fat and starchy compounds were found to be a reservoir of *Serratia marcescens* and other pigment producing microbes. Fifteen different food samples like bread, cheese, creams, cakes etc. were collected from different bakeries and food industries of Baramati, Pune, India. In sterile plastic bags, carry to laboratory and store under aseptic condition in $4^0$C until use.

#### Milk sample

Twenty different Milk samples were used in this study mainly obtained from Dynamix dairy, Gokul dairy and Vadilal dairy of Baramati, Pune and Ahmednagar respectively. This milk samples were collected before pasteurization treatment in pre-sterilized container and sore in refrigeration temperature until use.

#### Clinical samples

Three clinical samples viz. Blood, Urine and Sputum were screen for isolation of potent Prodigiosin producing microorganism. All three clinical samples used in this study were obtained from rural hospital Rui (Baramati, Pune, India)

#### Soil sample

Soil samples were collected from 23 different regions of the Pune and Ahemdnagar district of Maharashtra, India for the isolation of Prodigiosin producing microorganism.

Four samples were collected from the vicinity of the Baramati region. Ten samples were collected from several gardens and lawns of Ahemdnagar city. Remaining Nine samples were obtained from sangam regions of river Godavari and Bhima from Newasa and Daund (Maharashtra) respectively.

The soil samples were collected from 8-10cm depth using a sterile spatula and transferred to pre autoclaved sterile glass bottles with rubber stoppers. All samples were brought to the laboratory and stored under refrigeration temperature. (Nageswaran *et al.,* 2005).

#### Water sample

Twenty five different water samples (Potable water and waste water) were collected from 25 different sites of Pune, Ahemdnagar and Aurangabad district of Maharashtra, India. Ten potable water samples were collected from Pravara sangam (Godavari River, Ahemdnagar, Maharashtra) and from Ujani dam (Solapur, Maharashtra). Then six samples were collected from sangam region of Bhima river (Daund, Maharashtra). Five samples were collected from effluent disposal plants of Dynamix dairy (Baramati, Pune, India) and MIDC of Ahemdnagar and Aurangabad. The rest of the samples (Four) were obtained from ponds, water tanks and rain water harvesting reservoirs of households (Baramati, Pune). The samples were collected from the sub-surface level at about 10cm-15cm depth by dipping pre-autoclaved sterile glass bottles and opening their stoppers under water. They were then tightly fastened and labeled. The samples were then brought to the laboratory and stored under refrigeration temperature. (Nageswaran *et al.,* 2005).

### Enrichment and isolation of pigment producing bacteria

For the enrichment of microorganisms, one gram/ml of each of five samples were inoculated in nutrient broth aseptically and incubated at 37ºC for 5-6 days in rotary shaker incubator. One gm from each of the five respective samples was mixed with 9 ml sterilized Ringer's solution and the suspension was then serially diluted up to $10^{-8}$ dilution. From the diluted suspension 0.1ml from $10^{-4}$ onward was transfer onto the sterile Luria-Bertani agar medium plates by performing four quadrants method. This plate then incubated at 37±2˚C for 5 days, because Prodigiosin is a secondary metabolite and red colored colonies of microorganism are generally visible in the stationary phase of growth. (Nageswaran *et al.,* 2005).

After incubation the plates were observed for the presence of red colored colonies. Isolated red-pigmented colonies were restreaked on same media to get pure culture.

### Screening of pigment producing bacteria

Out of eighty six samples of five different types, potential Prodigiosin producing Bacteria were isolated that produced distinct reddish-orange color colonies and were selected to obtain pure cultures. Selected screened isolates were maintained at 4˚C and subculture periodically for subsequent experiments.

### Production, extraction and characterization of produced pigment

#### Production of pigment

The Selected high yielding strains of *Serratia marcescens* (YP 2) were used for large scale production of Prodigiosin by using Peanut broth .0.1 ml suspension of obtained sixteen isolates were inoculated in 100ml peanut broth for methanol extraction and spread on petriplate for petroleum ether extraction. Inoculated flask and plate were incubated at $30^0$C for 24 h.

**Extraction of pigment**

After incubation Prodigiosin was extracted, for the extraction purpose methanol and petroleum ether extraction method was used.

**Methanol extraction method**

The culture broth of 500 ml contain pigment was mixed with an equal volume of methanol and kept the mixture in rotary shaker for 20 to 30 min. The mixture was then pours into centrifuge tube and shake vigorously using vortex mixture. Then centrifuge at 1000 rpm for 10 min.

The supernatant was collected and filtered through a Wattman filter paper. The filtrate was concentrated using a rotary evaporator and later extracted with 3.0 M chloroform. The chloroform phase was then collected and re-concentrated using evaporator to obtained crude powdered pigment.

**Petroleum ether method**

The culture growth was scrape out from nutrient agar plate and added in sterile centrifuge tube containing minimum amount of sterile saline. This was then centrifuged at 3000 rpm for 20 min. The supernatant was then discarded and of 1N NaOH (two times volume of cell mass/palate) was then added to it. The tubes were kept in boiling water bath for 1 hour. Equal volume of absolute ethanol was then added to it. The solution was mixed well and again centrifuged at 3000 rpm for 20 min. After centrifugation the upper clear layer was collected in another test tube and equal volume of petroleum ether was added to it. The solution was mixed well. The tube was kept as it is for 10 minutes for the separation of two phases. The upper phase of petroleum ether was then collected in evaporating dish and kept in boiling water bath till the complete evaporation of petroleum ether. After complete evaporation of petroleum ether, fine powdered reddish colored pigment was obtained. The obtained pigment sample was then mixed with minimum volume of ethanol and stored in refrigerator at $4^0$C till use.

**Quantitative characterization of pigment**

After extraction of pigment produced by sixteen isolate, extracted pigment was quantified by using following method Optical density of the extracted pigment solution was determined at 535 nm (Williams et. al., 1960; Chen et. al., 2006). The total Prodigiosin (mg/L) was calculated according to the following formula (Venil, C. K. and Lakshmanaperumalsamy, P.)

$$TP\ (mg/L) = \frac{\text{A D V1}}{70700.0 \text{ X V2}}$$

Where TP denotes the total pigment yield (mg/L), A the absorbance of methanol extract at 535 nm, D the dilution ratio, V1 the volume of methanol added, 7.07 x 10 4 is extinction coefficient of Prodigiosin and V 2 is the volume of fermentative liquid.

On the basis of yield of pigment obtained four bacteria strain were selected for further study.

**Identification of potential isolates**

The selected four strains (CS-I, SS-II, SS-IV, WS-IV) of bacteria were identified by their morphological, biochemical characterization.

Bacterial isolates were characterized by using Bergey's Manual of Systematic Bacteriology **(**Holt *et al.*, 1989) and Benson's Microbiological Applications, Laboratory Manual in General Microbiology (Brown, 2007**)**, ( Nageswaran N., *et al*).

**Qualitative characterization of prodigiosin**

**Absorbance of pigment**

The extracted red pigment was used for determination of its maximum absorbance (Lambda max value). For this the pigment produced by each of the four isolate was exposed to different filters of colorimeter (450 to 670 nm) the absorbance of pigment at different wavelength was noted. The values of absorbance were plotted against respective wavelength and finally the λ max was calculated. (Davraj *et al*., (2009), Song *et al* (2005)).

**Thin layer chromatography**

For characterization of the extracted pigment thin layer chromatography was performed (in triplicates). For this, readymade strip of silica gel was used. The pigment sample was applied on it. The strip was kept in a solvent system containing methanol, ethyl acetate and chloroform (5:4:1). The sample was then allowed to run up to $3/4^{th}$ of the gel. After that, the strip was kept in oven for 5 minutes for drying. No developing reagent was used for observation of spot but instead the strip was observed under U.V. light for spot observation. Rf value of the spot was determined. The average Rf value from triplicate experiments was compared with the values obtained by other researchers (Davraj *et al*., (2009), Song *et al* (2005)).

**RESULTS & DISCUSSION**

**Isolation of pigment producing bacteria**

**Starchy food sample**

Fifteen different food samples like bread, cheese, creams, cakes etc. were used for isolation of Prodigiosin producing bacteria. Total 3 potent red pigment producer bacteria were found in these samples. Detail of sampling site, sample type and organism obtained were given in the table number one.

**Milk sample**

Twenty different Milk samples were used in this study mainly obtained from Dynamix dairy, Gokul dairy and Vadilal dairy of Baramati, Pune and Ahmednagar respectively. Milk was found to be the great source of this pigment producers, because wide variety of bacteria were found which having ability to produce many different types of color in milk. Out of different pigment producing bacteria isolated from milk samples two bacterial isolates which producing Prodigiosin like pigment were selected for further study.

**TABLE NO.1**: Detail of total number of isolate obtained.

| Sr. No | Sample Number | Sample Type | Total Viable Count (cfu X $10^6$/ml) | Total Number of Red Pigmented colony obtained | Isolate Number |
|---|---|---|---|---|---|
| 1 | | Starchy Food Sample | | | |
| | 3 | Bread | 1.23 | 1 | FS –I |
| | 7 | Bread | 2.48 | 1 | FS-II |
| | 15 | Cheese | 1.97 | 1 | FS-III |
| 2 | | Milk Sample | | | |
| | 11 | Dynamix | 1.25 | 1 | MS-I |
| | 17 | Dynamix | 0.065 | 1 | MS-II |
| 3 | | Clinical Samples | | | |
| | 2 | Urine | 0.0051 | 1 | CS-I |
| 4 | | Soil Sample | | | |
| | 6 | Baramati region | 3.58 | 2 | SS-IA,B |
| | 13 | Sangam region (Bhima) | 4.71 | 1 | SS-II |
| | 14 | Ahemdnagar City | 5.28 | 2 | SS-IIIA,B |
| | 19 | Sangam region (Godavari) | 2.19 | 1 | SS-IV |
| 5 | | Water Sample | | | |
| | 3 | Godavari river | 2.22 | 1 | WS-I |
| | 8 | Bhima river | 6.78 | 1 | WS-II |
| | 22 | Ujani dam | 5.21 | 1 | WS-III |
| | 24 | Nagar MIDC | 7.95 | 1 | WS-IV |

On the basis of yield of pigment obtained four bacteria strain namely CS-I, SS-II, SS-IV and WS-IV were selected for further study.

**TABLE NO.2:** Biochemical characterization of isolates

| | Isolate Number | | | |
|---|---|---|---|---|
| Morphological Characteristics | CS I | SS II | SS IV | WS IV |
| Cell shape | Cocci | Bacilli | Bacilli | Cocci |
| Arrangement | Tetrads | Tetrads | Singly & groups | Singly & groups |
| Spores | - | - | - | - |
| Motility | + | + | + | + |
| Gram character | Negative | Negative | Negative | Negative |
| Colony Morphology | | | | |
| Configuration | Round | Round | Round | Round |
| Margin | Entire | Entire | Entire | Entire |
| Elevation | Convex | Convex | Convex | Convex |
| Surface | Smooth | Smooth | Smooth | Smooth |
| Pigment | Orange | Orange red | Red | Red |
| Opacity | Opaque | Opaque | Opaque | Opaque |

**Clinical samples**

Three clinical samples viz. Blood, Urine and Sputum were screen for isolation of potent Prodigiosin producing microorganism. As *Serratia marcescens* is a common Prodigiosin producing bacterium which is a opportunistic pathogen commonly found in urinary tract, so one bacterial isolate were obtained from the clinical sample which were selected for further study

**Soil sample**

Enrichment and isolation of microbes from 23 soil samples from different sites of Pune and Ahmednagar of Maharashtra revealed the presence of various pigment producing bacteria. These microbes were found to produce various kinds of pigment total red color pigment producing viable bacterial count obtained from soil sample were given in the table No. one.

**Water sample**

For isolation of pigment producing bacteria from water sample, bacterial colonies producing the distinct red pigment on Luria Bertani agar was selected. Different water sample collected from 25 different sites of Pune, Ahmednagar and Aurangabad district of Maharashtra, India show the presence of wide variety of microorganism that having ability to produce different color pigments. Total viable bacterial counts from the water samples are summarized in Table one.

**TABLE NO.3:** Morphological characterization of isolates

| | Isolate Number | | | | | Isolate Number | | | |
|---|---|---|---|---|---|---|---|---|---|
| Biochemical Test | CS -I | SS-II | SSI-V | WS-IV | Biochemical Test | CS -I | SS-II | SSI-V | WS-IV |
| Ala-Phe-Pro Arylamidase | - | - | - | - | D-Tagatose | - | - | - | - |
| Adonitol | + | + | + | + | D-Trehalose | + | + | + | + |
| L-Pyrrolydonyl-Arylamidase | + | + | + | + | Citrate(Sodium) | + | + | + | + |
| L-Arabitol | - | + | + | + | Malonate | - | - | - | - |
| D-Cellobiose | - | - | - | - | 5-Keto-D-Gluconate | - | - | - | - |
| Beta-Galactosidase | - | + | + | + | L-Lactate alkalinization | + | + | + | + |
| H2S Production | - | - | - | - | Alpha-Glucosidase | - | - | - | - |
| Beta-N-Acetyl Glucosaminidase | + | + | + | + | Succinate Alkalinization | - | - | - | - |
| Glutamyl Arylamidase pNA | - | - | - | - | Beta-N-Acetyl Galactosaminidase | + | + | + | + |
| D-Glucose | + | + | + | + | Alpha-Galactosidase | - | - | - | - |
| D-Lactose | - | - | - | - | Phosphatase | - | - | - | - |
| Gamma-Glutamyl-Transferase | - | - | - | - | Catalase | + | + | + | + |
| Fermentation Glucose | + | + | + | + | Oxidase | - | - | - | - |
| Beta-Glucosidase | + | + | + | + | Casein hydrolysis | + | + | + | + |
| D-Maltose | - | - | - | - | Glycine Arylamidase | - | - | - | - |
| D-Mannitol | + | + | + | + | Ornithine Decarboxilase | + | + | + | + |
| D-Mannose | + | + | + | + | Lysine Decarboxylase | + | + | + | + |
| Beta-Xylosidase | - | - | - | - | L-Histidine assimilation | - | - | - | - |
| Beta-Alanine arylamidase pNA | - | - | - | - | Courmarate | + | + | + | + |
| L-Proline Arylamidase | + | + | + | + | Beta-Glucuronidase | - | - | - | - |
| Lipase | - | - | - | - | O/129 Resistance (comp. vibrio) | + | + | + | + |
| Palatinose | - | - | - | - | Glu-Gly-Arg-Arylamidase | + | + | + | + |
| Tyrosine Arylamidase | - | - | - | - | L-Malate assimilation | - | - | - | - |
| Urease | - | - | - | - | Ellman | - | - | - | - |
| D-Sorbitol | + | + | + | + | L-Lactate assimilation | - | - | - | - |
| Saccharose/Sucrose | + | + | + | + | | | | | |

**Biochemical characterization of isolates by analytical profile index** OR **API**

The results of morphological and biochemical analysis suggest that all the selected strains of bacteria are of *serratia marcescens*. For convenience all four strain are labeled as YP 1, YP 2, YP 3 and YP 4 respectively.

**Screening and selection of potent pigment producing organism**

Out of eighty six samples of five different types, Soil and water sample shows presence of many different types of bacteria having ability to produce red pigment. Total 16 different bacterial strains were isolated and screen out for further study. (3, 2, 1, 6 and 4 isolate were obtained from food, Milk, Clinical, Soil and Water sample respectively).

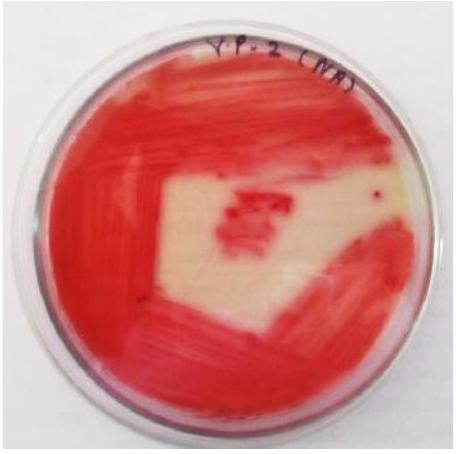

**FIGURE NO.1:** Growth of isolate YP 2 on Nutrient agar

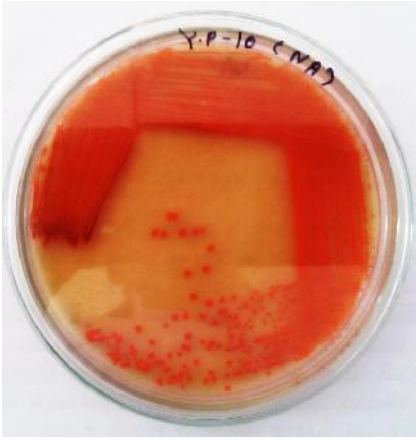

**FIGURE NO.2**: Growth of isolate YP 10 on Nutrient agar


**FIGURE NO.3**: Growth of isolate YP 14 on Nutrient agar


**FIGURE NO.4**: Growth of isolate YP 16 on Nutrient agar

**Identification of selected isolates**

The morphological and biochemical test (according to Bergey's manual) were used to identify the potential bacterial isolates (Holt *et al*., 1989). Standard protocol was used for performing each test (Brown, 2007). Results of morphological and biochemical tests of selected four isolate are shown in the table No. two and three respectively.

**Characterization of prodigiosin**

For the qualitative characterization of pigment 0.1 ml suspension of YP 2 was inoculated into 100 ml sterile peanut broth. After incubation pigment was extracted by using methanol extraction method and extraction product was subjected to spectroscopic and chromatographic studies.

**Absorbance of pigment**

The extracted red pigment from *serratia marcescens* (YP 2) was used for determination of its maximum absorbance (λ max) value. For this, the sample was exposed to different filters of colorimeter (450 to 670 nm). The absorbance value of pigment at respective wavelength was plotted and finally the λ max was calculated. It was found that, the red pigment has maximum absorbance (λ max) at 540 nm. This was compared with lambda max value of standard (538 nm). Song *et al.*, (2005) has extracted the Prodigiosin and found that, the extracted pigment has maximum absorbance at 536 nm. Davraj *et al*., (2009) has extracted the Prodigiosin from *Serratia Marcescens* (MTCC 97*) and found that, it has maximum absorbance at 540 nm.

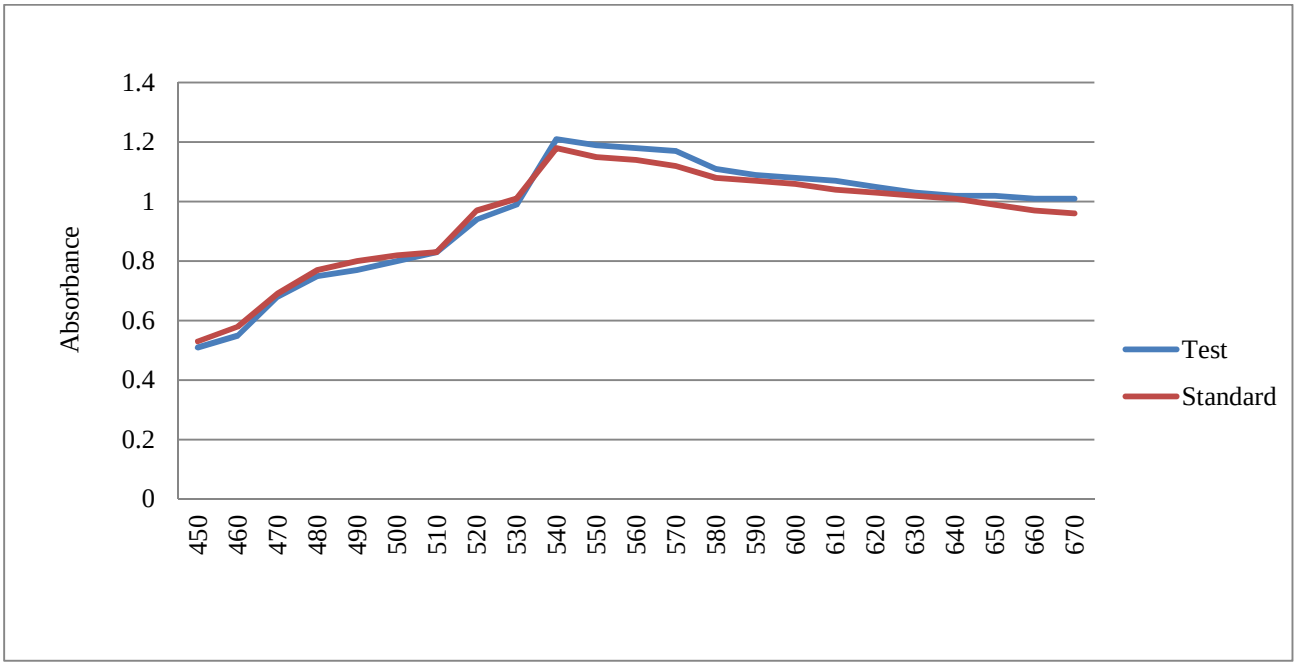


**FIGURE NO.5:** Absorbance of Extracted Red Pigmen

**Thin layer chromatography**

The extracted pigment was characterized by thin layer chromatography. For this, silica gel plates were used. After observing the silica gel under U.V. light, single, pink colored spot was observed. The Rf value of the spot was determined using formula. The average Rf value of pigment obtained from triplicate experiments was found to be 0.88.which compare with the standard pigment Rf value which was observed as 0.90. Davraj *et al*., (2009) have extracted Prodigiosin from *Serratia Marcescens* (MTCC 97*). They characterized the extracted pigment by TLC and found pink colored spot when visualized under UV light and having Rf value 0.9 to 0.95. Ramina M. *et al.*, (2009) reported the RF value of Prodigiosin 0.65. Samrot *et al*., (2011) found that, the isolated red pigment when subjected to TLC the obtained Rf value was 0.87.

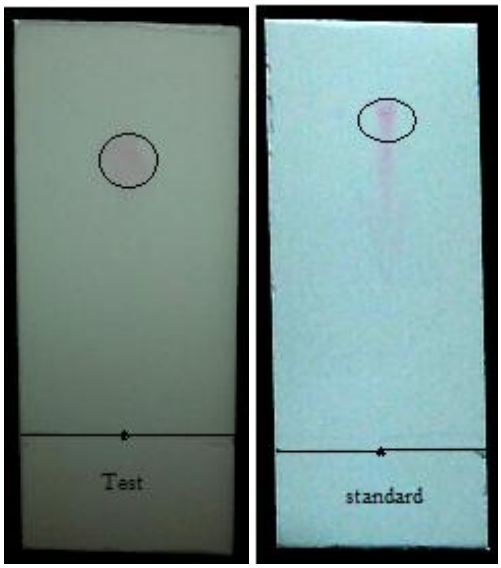


**FIGURE NO.6:** Thin layer chromatography of extracted pigment

From the results of absorbance (lambda max) of pigment and TLC (Rf value) it was concluded that, the red pigment produced by *serratia marcescens* (YP 2) was Prodigiosin.

**CONCLUSION**

The result thus obtained suggests that the Food, Clinical, Milk, soil and water samples collected from Pune, Ahmednagar and Aurangabad region of Maharashtra, India especially in and around the river banks is a thriving source for innumerable varieties of bacteria capable of producing various pigments. Some of which may prove to have many application in industries, environment pollution control and in medicine. Density of this organism in the environment is increases may be due to rise in human population and activities, which has significant impact on environment and on this organism. As well as day by day this bacterium are becoming resistant to wide variety of antimicrobial agents.

The pigment produced by selected strains of *serratia spp*. (YP2) was successfully characterized by using conventional methods.

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